



Karyotype characterization and nucleolar organizer regions of marsupial species (Didelphidae) from areas of Cerrado and Atlantic Forest in Brazil

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Abstract

The karyotypes of 23 specimens belonging to 16 species from nine genera of Brazilian marsupials (family Didelphidae) were studied. The animals were collected in eight localities of Cerrado or Atlantic Forest biomes in the states of Goiás, Tocantins and São Paulo. The karyotypes were analyzed after conventional Giemsa staining and silver staining of the nucleolar organizer regions (Ag-NORs). New karyotypic data were obtained for *Gracilinanus microtarsus* ($2n = 14$, FN = 24), *Marmosops paulensis* ($2n = 14$, FN = 24), *Micoureus paraguayanus* ($2n = 14$, FN = 20) and *Monodelphis rubida* ($2n = 18$, FN = 32) and are discussed in detail. The karyotypes of *G. microtarsus*, *M. paulensis* and *M. paraguayanus* include three large pairs of submetacentrics (pairs 1, 2 and 3) and a medium-sized metacentric or submetacentric pair 4. Pairs 5 and 6 are small submetacentrics in *G. microtarsus* and *M. paulensis* and acrocentrics in *M. paraguayanus*. *M. paulensis* presented a single Ag-NOR in pair 6 (6p6p), while *M. paraguayanus* exhibited multiple Ag-NORs in pairs 5 and 6 (5pq5pq6p6p). There was variation in size and morphology of the sex chromosomes among these species. *Monodelphis rubida* presented a karyotype with $2n = 18$ and FN = 32 composed of a large submetacentric pair 1, a medium-sized metacentric pair 2 and six pairs of submetacentrics (pairs 3 through 8). The X was a small acrocentric and the Y was dot-like. A single Ag-NOR bearing pair (5p5p) characterized *M. rubida*. Relevant karyotypic information was obtained for 19 specimens belonging to 12 species collected in areas sampled for the first time [*Caluromys lanatus* and *C. philander* ($2n = 14$, FN = 20), *Gracilinanus emiliae* ($2n = 14$, FN = 24), *Marmosa murina*, *Metachirus nudicaudatus* and *Micoureus demerarae* ($2n = 14$, FN = 20), *Monodelphis americana* ($2n = 18$, FN = 32) and *M. domestica* ($2n = 18$, FN = 20), and *Didelphis marsupialis*, *Philander frenata*, *P. opossum* and *P. sp* ($2n = 22$, FN = 20)]. Although the karyotypes were relatively conserved with respect to the morphology of the autosomes among species with the same diploid number, some differences regarding FN, sex chromosomes morphology and Ag-NORs patterns were detected.

Key words: marsupials, karyotypes, cytogenetics, Didelphidae, NORs.

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There are currently 331 recognized species of living marsupials assembled into seven orders, three of which occur in the American continent (Didelphimorphia, Paucituberculata and Microbiotheria). They represent an extreme example of karyotype conservation, which is observed in most living species from the Australian and American continents. Some marsupials present a $2n = 14$ karyotype, while others exhibit a karyotype with $2n = 22$ (Pagnozzi *et al.*, 2000; 2002; Svartman and Vianna-Morgante, 2003). The diploid number of $2n = 18$

also occurs, but so far it has only been observed in four species of the genus *Monodelphis* found in Brazil and Bolivia (Pagnozzi *et al.*, 2002; Carvalho *et al.*, 2002).

The most frequent $2n = 14$ karyotype has been traditionally considered ancestral to both American and Australian species (Rofe and Hayman, 1985; Hayman, 1990) and was suggested to have given rise to the highest diploid numbers through a series of centric fissions (Rofe and Hayman, 1985; Metcalfe *et al.*, 2004). An alternative hypothesis claimed that the ancestral karyotype was similar to that with $2n = 22$ and that centric fusions played a role on the origin of the derived karyotypes with lower diploid numbers (Svartman and Vianna-Morgante, 1998; Carvalho and Mattevi, 2000).

Didelphidae belongs to the order Didelphimorphia and is the most diverse American family of marsupials, with 19 genera and 103 species (Wilson and Reeder, 2005). About 25 Brazilian species of marsupials have already had their karyotypes reported (Yonenaga-Yassuda *et al.*, 1982; Casartelli *et al.*, 1986; Souza *et al.*, 1990; Pagnozzi *et al.*, 2000, 2002; Carvalho *et al.*, 2002; Svartman and Vianna-Morgante, 1999; 2003; Paresque *et al.*, 2004).

Karyological studies have demonstrated that the nucleolus organizer regions (NORs) and C-banding patterns represent important cytogenetic markers in conserved karyotypes because they reveal patterns that may characterize different species. According to Svartman and Vianna-Morgante (1999), marsupial karyotypes with the same diploid

numbers differed in the amount of pericentromeric constitutive heterochromatin and in the number and distribution of Ag-NORs. These differences among species may be useful in the taxonomy of Didelphidae.

We analyzed 23 specimens belonging to 16 species from nine genera of Didelphidae collected in eight different localities of Cerrado and Atlantic Forest in the states of São Paulo, Goiás and Tocantins (Brazil). The karyotypes were analyzed after conventional staining and after silver staining of the nucleolus organizer regions (Ag-NORs) (Table 1). We present data on 19 specimens belonging to 12 species collected in areas not previously surveyed (Table 1). We discuss in detail the new karyotypic data obtained for specimens of *Gracilinanus microtarsus*,

Table 1 - Chromosome data of Didelphidae from Brazil.

Species	Specimen/filed numbers ¹	Collection (locality and coordinates)	2n/FN	Sex	X	Y	Number of cells analyzed		NORs position	Number of NORs
							CS	AgS		
<i>Caluromys lanatus</i>	MM 11	UHE Corumbá IV (Luziânia, GO) 16° 15'09" S, 47° 57'01" W	14/20	M	SM	D	28	10	6p6p	2
	MM12		14/20	M	SM	D	37	-		
<i>Caluromys philander</i>	UNIBAN 2589	Biritiba-Mirim (SP) 23° 34'21" S, 46° 02'19" W	14/20	F	A	-	34	12	6p6p	2
	UNIBAN 2622		14/20	M	A	D	32	6	6p6p	2
	UNIBAN 2625		14/20	F	A	-	16	5	6p6p	2
<i>Gracilinanus emiliae</i>	MM 10	UHE Corumbá IV (Luziânia, GO)	14/24	M	SM	A	30	6	6p6p	2
* <i>Gracilinanus microtarsus</i>	UNIBAN 2602	Biritiba-Mirim (SP)	14/24	F	M	-	24	-	-	-
<i>Marmosa murina</i>	MM 13	UHE Corumbá IV (Luziânia, GO)	14/20	M	SM	A	30	22	6p6p	2
	MM14	UHE Peixe Angical (TO) 12° 01'30" S, 48° 32'21" W	14/20	M	SM	A	30	-	-	-
* <i>Marmosops paulensis</i>	UNIBAN 2307	Biritiba-Mirim (SP)	14/24	M	M	A	31	28	6p6p	2
<i>Metachirus nudicaudatus</i>	APC 1436	PESM (Caraguatatuba, SP) 23° 37'13" S, 45° 24'47" W	14/20	F	A	-	33	5	6p6p	2
<i>Micoureus demerarae</i>	UNIBAN 2074	Biritiba-Mirim (SP)	14/20	F	A	-	62	2	6p6p	2
	UNIBAN 2134		14/20	F	A	-	32	4	6p6p	2
* <i>Micoureus paraguayanus</i>	APC 1469	PESM (Caraguatatuba, SP)	14/20	F	A	-	32	10	5pq5pq 6p6p	6
<i>Monodelphis americana</i>	UNIBAN 2133	Biritiba-Mirim (SP)	18/32	F	A	-	14	-	-	-
<i>Monodelphis domestica</i>	MM 15	UHE Corumbá IV (Luziânia, GO)	18/20	M	A	A	15	-	-	-
	MM16	UHE Peixe Angical (TO)	18/20	F	A	-	30	-	-	-
* <i>Monodelphis rubida</i>	UNIBAN 2311	Biritiba-Mirim (SP)	18/32	M	A	A	17	25	5p5p	2
<i>Didelphis marsupialis</i>	MM 17	UHE Peixe Angical (TO)	22/20	F	A	-	25	-	-	-
<i>Philander frenata</i>	UNIBAN 3645	Serra da Cantareira (SP) 23° 32'51" S, 46° 38'10" W	22/20	M	A	A	31	17	5p5p7q 7q	4
<i>Philander opossum</i>	MM 18	PEAMP (GO) 16° 30'38" S, 49° 01'26" W	22/20	M	A	A	36	44	5p5p7q 7q	4
<i>Philander</i> sp	MCL 198	Boracéia (SP)	22/20	F	A	-	54	19	-	-
	MCL199	22° 11'35" S, 48° 46'44" W	22/20	M	A	A	52	-	-	-

* New karyotypic data described in the present study.

2n – diploid number; FN – fundamental (FN) numbers; M-metacentric; SM-submetacentric; A-acrocentric; D-dot-like; CS – conventional staining; AgS – silver nitrate staining, p- short arm; q- long arm.

UHE: Usina Hidrelétrica, PESM: Parque Estadual da Serra do Mar; PEAMP: Parque Ecológico Altamiro de Moura Pacheco (GO);

GO – Goiás, SP – São Paulo, TO – Tocantins.

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Marmosops paulensis, *Micoureus paraguayanus* and *Monodelphis rubida*.

Mitotic preparations were obtained from bone marrow and spleen following routine protocols. After Giemsa staining, at least 15 metaphases/individual were analyzed to establish the diploid ($2n$) and fundamental numbers (FN = number of autosome arms) and the chromosomes morphology. Metaphases were photographed with a 100X objective under a Leica CW 4000 or Zeiss Axiophot photomicroscope equipped with image capture systems and softwares for chromosome analyses (Karyo, Leica and Ikaros Karyotyping System, MetaSystems). After silver staining (Howell and Black, 1980), the Ag-NORs were analyzed in the maximum number of cells possible.

Three diploid numbers were found in the studied Didelphidae: (I) $2n = 14$ in *Caluromys lanatus*, *C. philander*, *Gracilinanus emiliae*, *G. microtarsus*, *Marmosa murina*, *Marmosops paulensis*, *Metachirus nudicaudatus*, *Micoureus demerarae*, *M. paraguayanus*; (II) $2n = 18$ in *Monodelphis americana*, *M. domestica* and *M. rubida*; and (III) $2n = 22$ in *Didelphis marsupialis*, *Philander frenata*, *P. opossum* and *P. sp.* A summary of the karyotypic data is presented in Table 1.

I) Karyotypes with $2n = 14$

Gracilinanus microtarsus and *Marmosops paulensis* presented karyotypes with $2n = 14$ /FN = 24 composed of three pairs of large submetacentrics (pairs 1, 2 and 3), a medium-sized submetacentric pair 4 and two pairs of small submetacentrics (pairs 5 and 6); the X chromosome was a small metacentric (Figure 1A and B). The Y chromosome was acrocentric in *Marmosops paulensis* (Figure 1B). The karyotypes reported here for *G. microtarsus* and *M. paulensis* are very similar, despite the difference in the size of the X chromosome, larger in *G. microtarsus* than in *M. paulensis*. (Figure 1A and B).

The karyotype of a female *Gracilinanus microtarsus* from Biritiba-Mirim, São Paulo (Table 1), differed from specimens previously collected in the state of Rio Grande do Sul and also from individuals of *G. agilis* and *G. emiliae* trapped in Goiás and Minas Gerais (Carvalho *et al.* 2002; this work). While pair 4 was submetacentric and the X chromosomes were metacentric in our specimen of *G. microtarsus*, pair 4 was metacentric in the specimens of *Gracilinanus* previously reported and the X chromosome was a submetacentric in *G. microtarsus* and *G. agilis*. Although marsupial karyotypes have been traditionally considered conservative, the present results indicate that some degree of interspecific karyotype variation occur in Didelphidae. In *Gracilinanus*, Ag-NORs were located on the short arms of pair 6 in specimens of *G. microtarsus* and *G. emiliae*, but there are no data for *G. agilis* (Carvalho *et al.* 2002).

The karyotype of *Marmosops paulensis* is reported herein for the first time. The submetacentric pair 4 of *M.*

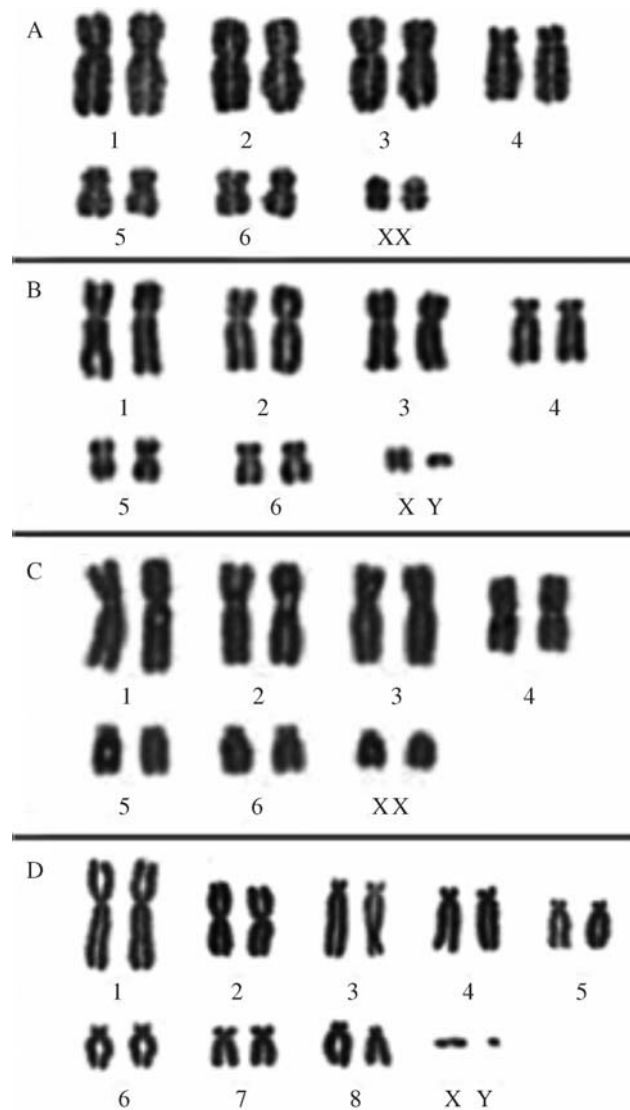


Figure 1 - Karyotypes of four species of Didelphidae after conventional staining: (A) *Gracilinanus microtarsus* female ($2n = 14$, FN = 24); (B) *Marmosops paulensis* male ($2n = 14$, FN = 24); (C) *Micoureus paraguayanus* female ($2n = 14$, FN = 20); (D) *Monodelphis rubida* male ($2n = 18$, FN = 32).

paulensis differed from the same pair described as metacentric in specimens of *M. incanus* from Espírito Santo and Bahia (Paresque *et al.*, 2004; Pagnozzi *et al.*, 2002) and in *M. parvidens* from Mato Grosso (Pagnozzi *et al.*, 2002). The X and Y chromosomes of *M. paulensis* are metacentric and acrocentric, respectively, as were those observed in *M. parvidens* (Pagnozzi *et al.*, 2002) and *M. incanus* (Paresque *et al.*, 2004). The Ag-NORs were located at the telomeres of the short arms of pair 6 in *M. paulensis* (Figure 2A; Table 1). This result agrees with those reported by Svartman and Vianna-Morgante (2003) and Carvalho *et al.* (2002) for *Marmosops incanus* and *M. parvidens* from Goiás and Bahia, Brazil.

Micoureus paraguayanus exhibited $2n = 14$ and FN = 20 and a karyotype with three pairs of large submeta-

centrics (pairs 1 through 3), one pair of medium metacentrics (pair 4) and two small acrocentric pairs (pairs 5 and 6; Figure 1C). The X chromosome was a small acrocentric. This karyotype is identical to that previously described for specimens of *M. demerarae* collected in the Amazon and in the states of Pernambuco, Mato Grosso, Bahia and São Paulo (Casartelli *et al.*, 1986; Souza *et al.*, 1990; Pagnozzi *et al.*, 2000; Svartman and Vianna-Morgante, 1999). A karyotype with $2n = 14$ and $FN = 24$ was reported in five individuals identified as *M. demerarae* trapped in localities of Goiás and Rio Grande do Sul (Carvalho *et al.* 2002). We believe that the difference in the reported FNs is due to distinct degrees of chromatin condensation of the short arms of pairs 5 and 6, which were considered as binned by Carvalho *et al.* (2002).

According to Voss and Jansa (2003), the specimens identified as *Micoreus demerarae* collected in Rio Grande do Sul and analyzed by Carvalho *et al.* (2002) were misidentified and were actually *M. paraguayanus*. We cannot discard the possibility that other previously karyotyped specimens of *M. demerarae* were also misidentified. Nevertheless, the present paper is the first to describe the karyotype and the Ag-NORs distribution in a specimen identified as *M. paraguayanus*.

Micoureus paraguayanus was the only species from our sample with more than two Ag-NORs. Besides the

Ag-NORs at pair 6, this species also had Ag-NORs at the telomeres of both chromosome arms of pair 5 and there was variation in the number of Ag-NORs per cell. Out of ten cells analyzed, two presented five Ag-NORs: on the telomeres of the long arms of one homologue of pair 5, on both telomeres (long and short arms) of the other element of pair 5 and on the telomeres of the short arms of pair 6 (5q5pq6p6p). The remaining eight cells presented six Ag-NORs (5pq5pq6q6p) (Figure 2B; Table 1). This 5pq5pq6q6p Ag-NORs pattern was also reported in specimens of *M. demerarae* from Rio Grande do Sul (Carvalho *et al.*, 2002) and by Svartman and Vianna-Morgante (2003), who described six positive signals after FISH with a ribosomal probe and after silver staining in a single specimen of *M. demerarae* from an unknown Brazilian locality. It is important to point out that the two specimens of *M. demerarae* herein studied were collected in a locality not previously sampled (Biritiba-Mirim) and exhibited a single pair with an Ag-NOR (pair 6; Table 1), differing from the multiple Ag-NORs (four to six sites) reported in the literature for specimens collected in the Amazon (Casartelli *et al.*, 1986) and in the states of Pernambuco (Souza *et al.*, 1990), Goiás and Rio Grande do Sul (Carvalho *et al.*, 2002). Nevertheless, we analyzed the Ag-NORs in only six cells (Table 1) and more cells have to be analyzed in order to confirm this result.

The specimens of *Marmosa murina* from areas of Cerrado in Goiás and Tocantins presented $2n = 14$ and $FN = 20$ (Table 1), a karyotype identical to the one found in specimens from Pernambuco (Souza *et al.*, 1990) and Espírito Santo (Paresque *et al.*, 2004). However, karyotypes with $2n = 14$ but presenting $FN = 22$ and 24 were described for specimens from Tocantins [Porto Nacional: $FN = 22$ (Lima, 2004) and $FN = 24$ (Carvalho *et al.*, 2002)], Amapá ($FN = 24$, Carvalho *et al.*, 2002) and Goiás (Serra da Mesa: $FN = 24$, Carvalho *et al.*, 2002). The difference in FNs among different specimens of *M. murina* reported in the literature reflects distinct classifications of pairs 5 and 6, which were considered binned by Carvalho *et al.* (2002) and Lima (2004). Moreover, while the X chromosome was a submetacentric in the specimens of *M. murina* studied herein (Table 1), only acrocentric X chromosomes were reported previously (Souza *et al.*, 1990; Carvalho *et al.*, 2002; Paresque *et al.*, 2004).

II) Karyotype with $2n = 18$

The karyotype of *Monodelphis rubida* had $2n = 18$ and $FN = 32$, and the eight pairs of autosomes included one large pair of submetacentrics (pair 1), one medium-sized metacentric pair (pair 2) and six pairs of submetacentrics (pairs 3 through 8). The X chromosome was a small acrocentric and the Y was dot-like (Figure 1D).

Although all the species of *Monodelphis* already described presented karyotypes with $2n = 18$, there was variation in the FNs among and within species. For instance,

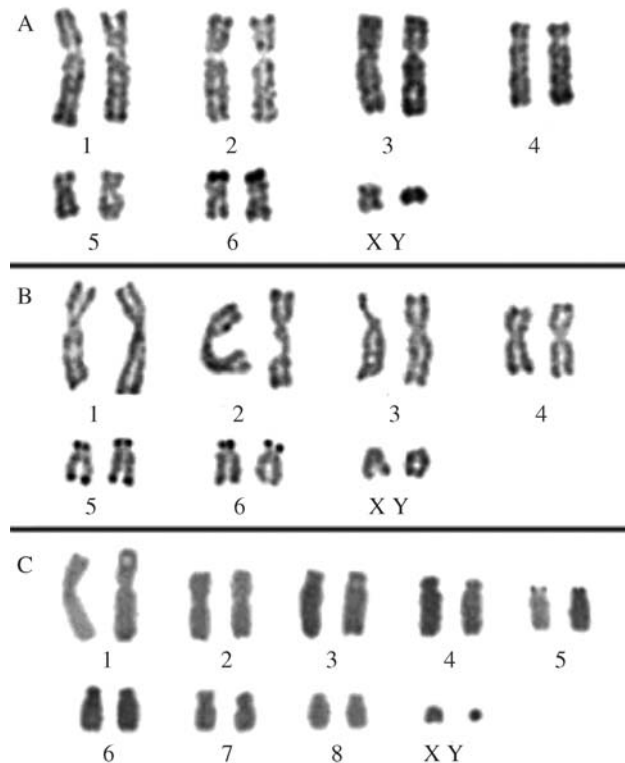


Figure 2 - Ag-NORs in the karyotypes of three species of Didelphidae: (A) *Marmosops paulensis* male (Ag-NORs in 6p6p); (B) *Micoureus paraguayanus* female (Ag-NORs in 5pq5pq6q6p); (C) *Monodelphis rubida* male (Ag-NORs in 5p5p).

karyotypes with FN = 20 and FN = 28 (sampled in localities of Goiás) were both described for specimens of *M. domestica* (Svartman and Vianna-Morgante, 1999; Carvalho *et al.* 2002, respectively). The lack of information on the collection sites for the specimens analyzed by Svartman and Vianna-Morgante (1999) prevents further considerations of a possible geographical variation within this species. A fundamental number as high as 30 was described in specimens of *M. domestica* from Espírito Santo (Paresque *et al.*, 2004), *M. kunsii* collected in localities of Goiás and *M. brevicaudata* from Roraima and Pará (Carvalho *et al.*, 2002). A karyotype similar to that found in *M. rubida* (present work), with exclusively biarmed chromosomes and FN = 32, also characterizes *M. dimidiata* from Rio Grande do Sul (Carvalho *et al.*, 2002) and specimens of *M. americana* from São Paulo (Biritiba Mirim; Table 1) and from Espírito Santo (Paresque *et al.*, 2004).

M. rubida exhibited a single Ag-NOR on the telomeres of the short arms of pair 5 (Figure 2C; Table 1). An identical pattern was found in two species of *Monodelphis* (*M. kunsii* and *M. dimidiata*) from Goiás and Rio Grande do Sul, but a different pattern was reported in *M. brevicaudata* (from the states of Roraima and Pará) and *M. domestica* (Goiás), in which Ag-NORs were observed on the telomeres of the short arm of the X chromosome (Carvalho *et al.*, 2002). Moreover, in specimens of *M. domestica* analyzed by Svartman and Vianna-Morgante (2003) four NORs (5p5p, XpXp) were reported in females and three (5p5p, Xp) in males.

A significant variation is observed in the FNs reported for specimens of *Monodelphis domestica* from different localities in Brazil. Karyotypes with $2n = 18$ and FN = 20 occurred in specimens from two localities of Cerrado in Goiás and Tocantins (present study, Table 1) and in specimens analyzed by Svartman and Vianna-Morgante (1999, 2003; without information on locality). Karyotypes with FN = 28 and FN = 30 were described for specimens from Cerrado of Goiás (Serra da Mesa and Ipameri; Carvalho *et al.*, 2002) and areas of Atlantic Forest in Espírito Santo (Paresque *et al.*, 2004), respectively. The reports of FNs higher than 20 is due to subtelocentric chromosomes being considered as biarmed by several authors and also due to differences in chromatin condensation. The standardization of chromosome nomenclature in marsupial species would prevent the artificial differences that make up for the confusing literature, especially for species in which there is no clear evidence of geographical variation.

In this study we described the karyotypes of three species of Didelphidae from Brazil (*Marmosops paulensis*, *Monodelphis rubida* and *Micoureus paraguayanus*) and a new karyotype for *Gracilinanus microtarsus*. Our work also increased the number of localities surveyed for 12 marsupial species. Although there was conservation of the autosomes morphology among karyotypes of species with the same diploid number, some differences regarding FN,

morphology of the sex chromosomes and Ag-NORs distribution were detected, indicating that marsupial karyotypes are less uniform than believed.

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